THE UPTAKE OF PHYSOSTIGMINE BY THE RED BLOOD CELL

and

THE EFFECT OF FEEDING PHENOTHIAZINE AND PHENOTHIAZINE DERIVATIVES TO VITAMIN E-DEFICIENT RATS

by

Eva Elizabeth Dellert

THE UNIVERSITY OF ALBERTA

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By

EVA ELIZABETH DELLERT

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ABSTRACT

The uptake of physostigmine by the red blood cell has been studied and discussed. Some physostigmine was taken up by the red cell. Washing the erythrocytes after incubation removed any physostigmine which was bound by the red cell. Neither calcium acetate nor acetylcholine bromide increased the amount of physostigmine taken up by the red blood cell. Red cell "ghosts" bound less physostigmine than did the whole red cell.

The effect of feeding phenothiazine, phenothiazone, and phenothiazine-o-sulfoxide to vitamin E-deficient rats has been studied in respect to the dialuric acid hemolysis of the red cell and the cholinesterase level of the erythrocyte. The erythrocytes of tocopherol-deficient rats undergo hemolysis in the presence of dialuric acid while the red blood cells of normal rats do not. The cholinesterase level of red cells of vitamin E-deficient rats is lower than that of rats receiving vitamin E in the diet. Both phenothiazine and phenothiazone were capable of replacing alpha-tocopherol in the diet as shown by their ability to protect the erythrocytes against dialuric acid hemolysis and to maintain the cholinesterase level of the red blood cell. Phenothiazone-o-sulfoxide failed to replace vitamin E

in the diet in respect to the two criteria mentioned.

A study on the sodium and the potassium content of the red cells of vitamin E-deficient rats showed the concemtration of these two ions to be normal.

ACKNOWLEDGEMENT

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THE UPTAKE OF PHYSOSTIGMINE BY THE RED BLOOD CELL

INTRODUCTION

Greig and Holland (1) showed that the addition of physostigmine to a suspension of erythrocytes caused hemolysis of the cell with a change in the permeability of the cell to potassium and water. They thought this effect was due to an inhibition of the acetyl cholinesterase system in the cell. Taylor and Weller (2) showed that the incubation of human blood in a saline solution containing 1 to 10 mM physostigmine would lead to a continuing loss of potassium from the cell. Christensen and Riggs (3) thought that the physostigmine cation itself might be displacing significant amounts of inorganic cations. In connection with this theory they showed that higher concentrations of physostigmine could be observed within the cells than outside upon incubation with physostigmine. They demonstrated that physostigmine (or, as it is often called, eserine) reached a level about two times as high in mouse ascites carcinoma cells and in human erythrocytes, and three times as high in duck erythrocytes as in the suspending fluid. They also found that varying the concentration of the physostigmine from 5 to 50 mM did not greatly alter the dis-

tribution ratios.

Christensen and Riggs thought that the potassium leakage from the red blood cells was not caused by inhibition of cholinesterase because the concentrations required to cause measurable potassium transfer was at least ten times as great as those required to stop all cholinesterase action in the plasma. At the same time they showed that enough physostigmine could get into the cells to stop all cholinesterase action there.

The present investigation was designed to determine whether the physostigmine cation was bound at all by the red cell membrane since it seemed to be a rather large ion as far as penetrating the cell wall is concerned. Physostigmine was therefore incubated with red blood cells and the uptake by the cell was determined.

EXPERIMENTAL

Blood was taken from rabbits by means of cardiac puncture and heparin was added in order to prevent clotting. The cells were centrifuged and washed once with isotonic saline. 0.25 ml of cells were then incubated with physostigmine under various conditions. 10 and 20 mM. solutions of physostigmine sulfate in buffered saline were used. After incubation periods of one, two, and four hours at room temperature (20°C to 25°C) the cells were centrifuged, diluted with an equal volume of water and then nine volumes of 0.75 N. nitric acid were added in order to precipitate the protein (4). The filtrate upon centrifuging was used for the determination of physostigmine.

Determination of Physostigmine

Materials

1. M/8 phosphate buffer pH 7.4
   3.45 gm. monosodium phosphate
   14.2 gm. disodium phosphate
   to one liter with water.
2. 10% sodium hydroxide
3. 6 N. hydrochloric acid

Physostigmine was determined by a modification (3) of the method of Ellis, Plachte, and Straus (5). Aliquots

of the nitric acid extract were diluted five times with the calculated amount of sodium hydroxide required to neutralize the nitric acid present. 1 ml. portions of this solution were taken. One drop of 10% sodium hydroxide was added in order to develope the red color of rubroeserine (4): then an approximately equivalent amount of hydrochloric acid was added. The solution was then diluted to 5 ml. with M/8 phosphate buffer. The optical densities were measured at 485 mμ in a Beckman Model DU spectrophotometer. Physostigmine was estimated in the suspending fluids in a similar manner after the supernatant had been diluted ten times.

The stanard curve shown in Figure 1 was prepared by adding physostigmine to portions of the fluid prepared from control determinations which were not incubated with physostigmine.

Uptake of Physostigmine by Erythrocytes

Materials

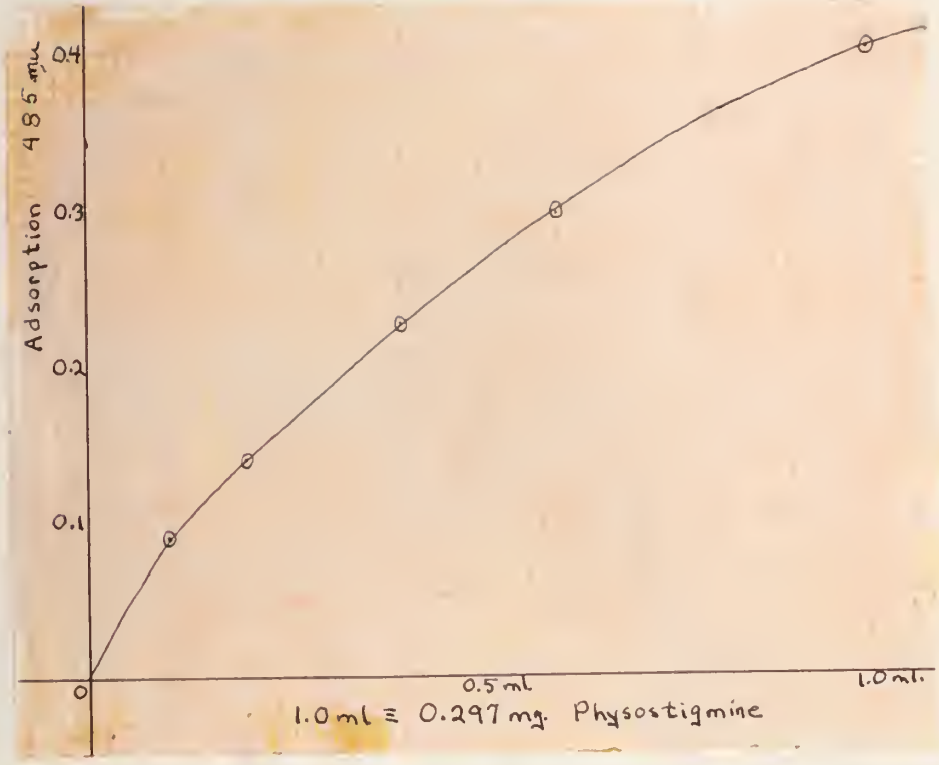
1. Phosphate buffered saline pH 7.4
   800 ml. 0.95% sodium chloride
   200 ml. M/8 phosphate buffer pH 7.4
2. Barbital buffered saline pH 7.3
   4.5 gm. sodium chloride
   0. 515 gm. sodium barbital

1.84 ml. 0.1 N. sulfuric acid

to 100 ml. with water. Diluted 1:5 daily as needed.

The determinations of the amount of physostigmine bound by the red cells were carried out in phosphate-buffered saline. The erythrocytes were incubated in the presence of physostigmine as described previously. After incubation they were centrifuged and the amount of physostigmine in the cells and the amount present in the supernatant fluid was determined. Replicate determinations were within one percent.

FIGURE 1



Calibration Curve for the Determination of Physostigmine

Effect of Washing Cells After Incubation

Cells were incubated with physostigmine and after incubation they were washed three times with phosphate-buffered saline. The physostigmine content of the red cells was determined as above.

Effect of Adding Calcium Ions

Erythrocytes were incubated in barbital-buffered saline containing calcium (approximately 0.01 M.), in the form of calcium acetate, and physostigmine.

Effect of Adding Acetylcholine Bromide

Washed red cells were incubated in the presence of physostigmine and acetylcholine bromide (0.01 M.).

Uptake of Physostigmine by Red Cell "Ghosts"

Red cell "ghosts" were prepared by diluting 4 ml. of erythrocytes to 500 ml. with water (6). The "ghosts" were washed with phosphate-buffered saline and were suspended in phosphate-buffered saline. They were incubated with physostigmine in the same manner as were the red cells. The determination of physostigmine was carried out in the same way.

RESULTS

Red cells incubated with physostigmine for the two and four hours showed no appreceable increase in the amount of physostigmine absorbed over those cells incubated with physostigmine for one hour. For this reason the results reported are all based on an incubation period of one hour.

Varying the concentration of the physostigmine from 10 mM. to 20 mM. showed no effect on the distribution ratios and hence the results reported are based on incubation in the presence of 10 mM. physostigmine sulfate.

The distribution ratios based on the content of physostigmine in mg./ml. of the cells and the supernatant fluid are shown in Table I. The effects of the various factors on the uptake of physostigmine are shown in the same table.

Physostigmine did not appear to be greatly concentrated in the cells. There was some uptake but the distribution ratios showed no noticeable defference between the supernatant fluid and the erythrocytes.

When the cells were washed with phosphate-buffered saline after incubation with physostigmine, no physostigmine could be detected in the cells.

When the cells were incubated in the presence of physostigmine and calcium ions there was a slight increase in the amount of physostigmine bound by the cells but the dis-

TABLE I

Erythrocytes only 6 Determinations	0.01 M. Ca present 8 Determinations	0.01 M. AChBr. present 4 Determinations	"Ghosts" 8 Determinations
0.96 ± 0.1	1.23 ± 0.15	0.95 ± 0.15	0.48 ± 0.16

Distribution ratios mg./ml. of cells to the supernatant fluid in respect to the amount of physostigmine present after incubation with 10 mM. physostigmine sulfate for one hour at room temperature.

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tribution ratios were still very close to one. When, as was done in one experiment, the cells were washed with phosphate-buffered saline after incubation with physostigmine and calcium acetate there was no physostigmine left in the cells.

Acetylcholine bromide also seemed to play no role in increasing the physostigmine uptake of the cell.

The experiments on incubating red cell "ghosts" with physostigmine seem to indicate that less physostigmine is

absorbed by the "ghosts" than by the whole red cells. Unfortunately there is no really accurate way of expressing the distribution ratio when "ghosts" are used. The results shown in Table I are based on the amount of packed "ghosts" which were used. This of course does not take into account any residual hemoglobin which might be present. It can only be said that "ghosts" did not appear to contain as much physostigmine after incubation with 10 mM. physostigmine sulfate as did erythrocytes.

DISCUSSION

The results would seem to indicate that some physostigmine is absorbed by the red cell when it is incubated in phosphate-buffered saline containing physostigmine. Although the distribution ratios obtained are by no means as high as those obtained by Christensen and Riggs (3) it cannot be said that their work has been in any way disproved.

The fact that when the erythrocytes are washed the physostigmine seems to be very easily removed would seem to indicate that if physostigmine is bound by the red cell it is a very weak binding. It is quite possible that it is a diffusion mechanism even though the size of the physostigmine cation would be thought large enough to prevent its permeation through the red cell membrane.

Calcium has only a slight effect on the distribution ratio and since the ratio is increased to such a small extent it might be said that the effect of calcium is negligible.

When acetylcholine bromide was added to the incubation solution there appeared to be no effect whatsoever on the amount of physostigmine bound by the cell.

This would lead to the hypothesis that acetylcholine and quite possibly the cholinesterase system is not extremely important in the uptake of physostigmine by the red cell. This would tend to agree with the theory put forth by Christensen and Riggs (3) who believed that the loss of potassium from the cells when incubated with physostigmine was due to an uptake of physostigmine.

Since physostigmine was taken up even less by red cell "ghosts" than by whole erythrocytes it would appear that physostigmine was not bound by the red cell membrane to any appreciable axtent. However the distribution ratios for the "ghosts" cannot be determined very accurately and for this reason the ratios shown in Table I may not be too true a picture of the amount of physostigmine absorbed by the "ghosts".

SUMMARY

1. After incubation of erythrocytes in the presence of physostigmine sulfate there was no appreciable concentration of the physostigmine in the red cells.

2. Washing the cells after incubation with physostigmine removed that which had been taken up by the red cell.

3. Neither calcium acetate nor acetylcholine bromide when added to the incubating solution containing physostigmine increased the amount of physostigmine bound by the cell.

4. "Ghosts" were shown to bind less physostigmine than did the whole red blood cells.

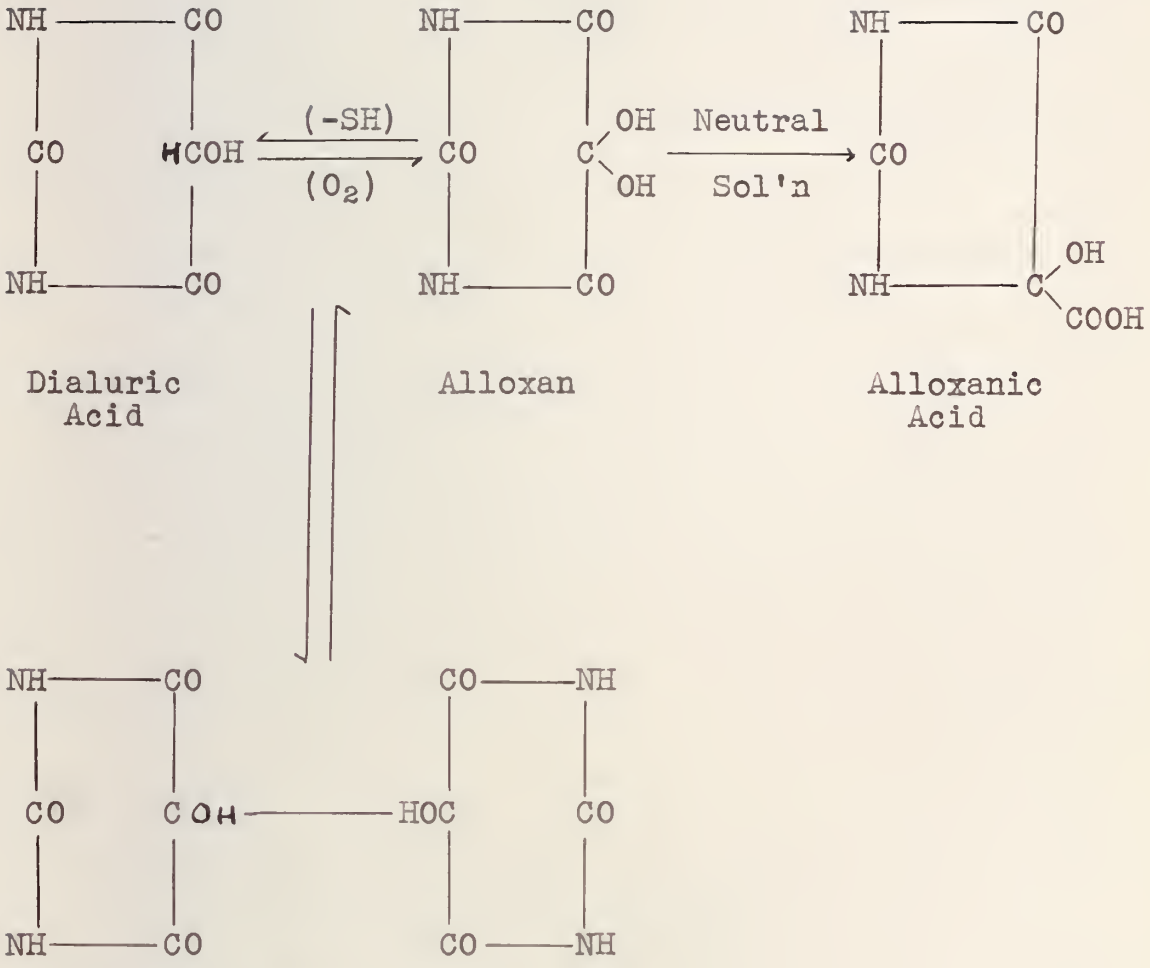
THE EFFECT OF FEEDING PHENOTHIAZINE AND PHENOTHIAZINE DERIVATIVES TO VITAMIN E-DEFICIENT RATS.

INTRODUCTION

Work on the dialuric acid hemolysis of the red blood cells of vitamin E deficient rats began with an experiment published by Houssay and Martinez in 1947 (7). They published a report concerning the protection of animals against the toxic effects of alloxan in the production of alloxan diabetes. They found that there was a longer survival in animals receiving a diet in which vegetable oils replaced the lard. György and Rose (8,9) wondered if vegetable oil was better than lard because of its vitamin E content. The hemolytic activity of alloxan in tocopherol deficient rats was first noticed at autopsy: the kidneys of these rats which had been injected with alloxan were completely engorged with blood (8).

Later work showed that dialuric acid, the product of the mild reduction of alloxan, was the active product (9). Alloxan as such disappears from the blood in a few minutes and for this reason it was thought that a derivative of alloxan might be the effective agent.(9) Alloxan, alloxanic acid, dialuric acid, and alloxantin,

FIGURE 2



Alloxan and Related Compounds (9).

which are all related compounds, were tried. Formulas and relationships between the various compounds are shown in Figure 2. Dialuric acid was found to be the only derivative which was effective to any extent both *in vivo* and *in vitro* and it was thought that hemolysis due to either alloxan or alloxantin was probably due to the formation of dialuric acid.

It has been found that alpha-tocopherol is the best protective agent in dialuric acid hemolysis with beta-, gamma-, and delta-tocopherols following in the order given (10). The prevention of hemolysis by vitamin E cannot be explained as a reaction between tocopherol and dialuric acid. Tocopherol is admittedly a reducing agent but it is a mild one and strong reducing agents are required for the reduction of dialuric acid. The explanation is thought to lie in the antioxidant properties of alpha-tocopherol (11). Dialuric acid is readily autooxidizable and it is thought likely that some molecule or radical formed during the oxidation of dialuric acid is the actual hemolyzing agent. It is beleived that the production of this hemolyzing agent may be prevented by tocopherol.

The antioxidant effect of alpha-tocopherol has been studied extensively by Dam and his co-workers. Dam thought that possibly the antioxidant effect was not specific for vitamin E but that other antioxidants would show some

vitamin E activity (12). They found that such substances as thiodiphenylamine (phenothiazine), antabuse, and methylene blue could counteract certain consequences of feeding a vitamin E-deficient diet high in cod liver oil. Amongst the symptoms of vitamin E-deficiency prevented by antioxidants were: maintenance of vitamin A storage in the liver (13), prevention of sterility in rats (14), incisor depigmentation, and coloration and peroxidation of the fat tissue in rats (12), and the prevention of dialuric acid hemolysis in rat erythrocytes (15). Christensen and Dam (15) found that the feeding of methylene blue at the level of 0.126% as an addition to a rat diet lacking tocopherols and containing lard and cod liver oil affords a marked though not complete protection against dialuric acid hemolysis.

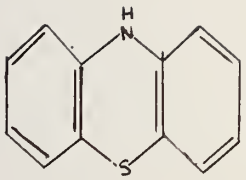
Moore,Sharman, Ward, and Heard (17, 18, 19) have disputed to some extent the findings of Dam and his co-workers. According to Moore, Sharman, and Ward (17) methylene blue will show some protection against vitamin E-deficiency symptoms such as depigmentation of the teeth but they found that the erythrocytes of animals fed methylene blue were no more resistant to hemolysis than were the red cells of tocopherol-deficient rats. However in a later paper Moore, Sharman, and Ward (19) stated that there seemed to be

some antioxidants which would prevent brown discoloration of the uterus, a common tocopherol-deficiency symptom. They found that both thiodiphenylamine and Bindschedler green had tocopherol action while such compounds as thionine, methyl violet, phenylene blue, methyl red, and Janus green had none. They thought that there must be required more than just the property of anti-oxidation by a compound in order to replace tocopherol in the diet.

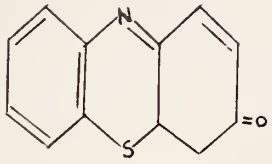
Thiodiphenylamine, or phenothiazine, which has been demonstrated both by Dam and his co-workers and by Moore and his co-workers to show some ability to replace vitamin E in the diet, is known to be an effective antioxidant. Because of its antioxidant properties, phenothiazine has been used in the lubricating industry (20).

It was decided by us to see if phenothiazine and two of its oxidized derivatives, phenothiazone and phenothiazine-o-sulfoxide, could replace alpha-tocopherol in the diet in relationship to the prevention of dialuric acid hemolysis. The structural formulas for phenothiazine, phenothiazone, and phenothiazine-o-sulfoxide are shown in Figure 3.

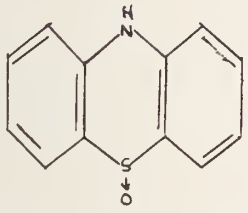
FIGURE 3



PHENOTHIAZINE



PHENOTHIAZONE



PHENOTHIAZINE-O-SULFOXIDE

Structural Formulas for Phenothiazine, Phenothiazone, and Phenothaizine-o-sulfoxide.

At the same time a study of the cholinesterase level of the red cells of the animals was carried out. It has been shown that there is a decrease in the cholinesterase content of the tissues in Vitamin E-deficiency (21) implying association of vitamin E and acetyl cholinesterase. In addition the dialuric acid

hemolysis of the cells of tocopherol-deficient rats has been shown to be a function of the cell itself and not a plasma factor (9). Since the cell was known to be altered the possibility of a change in the cholinesterase content of the cell was investigated. Determinations of the sodium and the potassium content of the red cells was carried out in order to determine whether the cation concentration of the cells of vitamin E-deficient animals was noticeably changed.

EXPERIMENTAL

Materials

The pure phenothiazine used in the diets and in the preparation of the derivatives, phenothiazone and phenothiazine-o-sulfoxide, was prepared by Mr. Gordon Allenby. Commercial phenothiazine (Merck) was recrystallized from benzene and after being washed with hexane was dried *in vacuo*.

Phenothiazone was prepared by the method of Pummerer and Gassner (22).

Phenothiazine-o-sulfoxide was prepared by a modification (22) of the method of Barnett and Smiles (23).

Experimental animal

Female albino rats weighing approximately 100 grams were used in all experiments. Groups of rats on the various diets were housed together. Blood was taken when the animals were killed by decapitation.

Diets

I Vitamin E-deficient diet (10)

Casein	20	parts	by	weight
Lard	8	"	"	"
Sucrose	66	"	"	"
Salt mixture U.S.P. #2	4	"	"	"
Cod liver oil	2	"	"	"

for each Kilogram of diet

Thiamine chloride	2.5 mg.
Calcium pantothenate	12 mg.
Pyridoxine	2.5 mg.
Riboflavin	3 mg.

II Vitamin E-containing diet

To the above diet was added 25 mg. of alpha-tocopherol per Kilo. of diet.

III Phenothiazine diet

To the regular vitamin E-deficient diet was added phenothiazine at a level of 0.126%.

IV Phenothiazone diet

Phenothiazone was added to the vitamin E - deficient diet at a level of 0.126%.

V Phenothiazine-o-sulfoxide diet

Phenothiazine-o-sulfoxide was added to the

vitamin E-deficient diet at a level of 0.126%.

Methods

Dialuric acid hemolysis

Reagents

Phosphate buffer (24)

25 ml. of 0.2 M. potassium monophosphate

19.7 ml. of 0.2 M. sodium hydroxide

water was added to the above to make the solution up to 100 ml. Sodium hydroxide was added if necessary to bring the buffer to the desired pH of 7.4. The final molarity was 0.05.

Dialuric acid

0.1% dialuric acid in phosphate buffer was made up fresh immediately before use.

0.9% sodium chloride

Procedure (10)

In each of three tubes was placed 0.25 ml. of a 1:20 suspension of red cells in 0.9% sodium chloride solution. To one tube was added 0.25 ml. of phosphate buffer: to the other tubes was added 0.18 ml. of phosphate buffer and 0.07 ml. of a 0.1% solution of dialuric acid. The

tubes were shaken and incubated at 37°C for 30 minutes. At the end of this time the tube without dialuric acid and one of those containing dialuric acid were diluted to 5 ml. with a mixture of equal parts of 0.9% sodium chloride solution and phosphate buffer. After careful mixing the tubes were centrifuged and the optical density of the supernatant was read in a Coleman photoelectric colorimeter at 540 mμ. The second tube containing dialuric acid was diluted to 5 ml. with distilled water and gave the value for the total hemoglobin. The reading of the blank corrected for any spontaneous hemolysis of the cells. The percentage hemolysis was calculated as follows.

$$\frac{\text{light absorption due to dialuric acid hemolysis}}{\text{light absorption due to water hemolysis}} \times 100 = \% \text{ hemolysis}$$

Cholinesterase determination

The method used was a modification of the electrometric method of Michel (25). The activity of the cholinesterase was expressed in microliters of acetic acid liberated by

0.1 ml. of washed packed cells in one-half hour at 37°C.*

Reagents

1. Buffer

Sodium barbitone	4.12 grams
Monobasic potassium phosphate	0.54 grams
Potassium chloride	44.73 grams

dissolved in 900 ml. of water and pH determined. Hydrochloric acid or sodium hydroxide was added until a pH of 8.10 was reached. The solution was diluted to one liter giving final concentrations of 0.02 M. sodium barbitone, 0.004 M. monobasic potassium phosphate, and 0.60 M. potassium chloride.

2. Substrate

0.11 M. acetylcholine bromide was the substrate used, being made up fresh daily.

3. 0.01% saponin, made up fresh daily.

4. 0.9% sodium chloride.

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* I am indebted to the Department of Physiology, University of Alberta for their work on the determination of cholinesterase in the red blood cell.

Procedure

Blood was collected from the rats into isotonic saline containing heparin. The cells were centrifuged and washed twice with 0.9% sodium chloride. 0.1 ml. of the washed packed cells was measured into 5 ml. of equal volumes of buffer and saponin solution. 0.5 ml. of acetylcholine bromide was added and exactly half a minute later the pH of the solution was noted on a Beckman pH meter using the small electrodes. The solution was incubated at 37°C for 30 minutes from the time the substrate was added and then the pH was again taken. The amount of acetic acid liberated was determined from a standard curve.

The standard curve (Figure 4) was determined as follows: 2.5 ml. buffer and 2.5 ml. saponin solution were used to determine the standard curve. Approximately 1.0 N. acetic acid (standardized) was added by means of a micro-burette. The pH was taken before and after the additions of acetic acid. The pH change was plotted against the microliters of acetic acid used. Since the dilution of the cells was great no correction was made for the buffering action of the cell proteins.

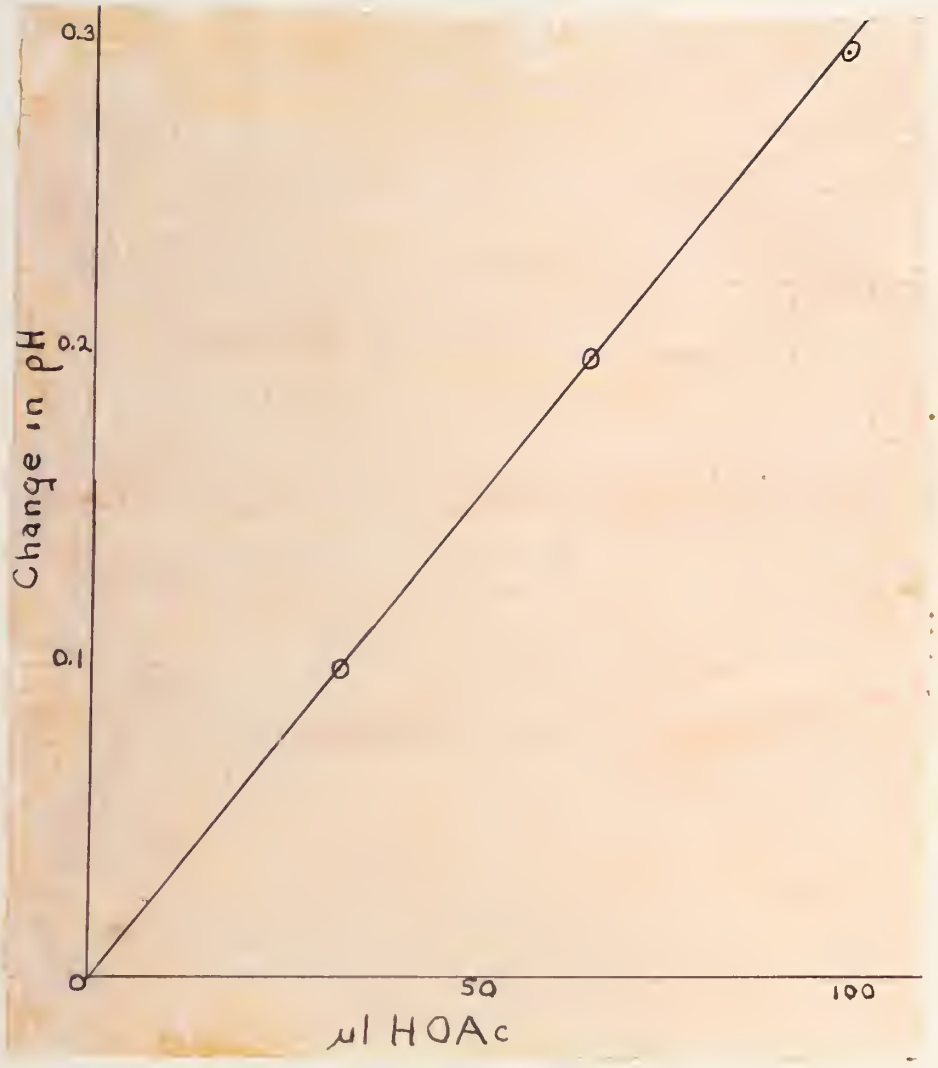
The cholinesterase activity of the erythrocytes was expressed as the microliters of 1.0 N. acetic acid liberated from the acetylcholine bromide by 0.1 ml. of washed

packed red blood cells.

Determination of sodium and potassium

The determination of the sodium and potassium content of the cells was carried out by Mr. Peter Solvonuk. Sodium and potassium were determined by means of a Beckman Model DU flame photometer. Blood was collected from the rats into 0.3 M. sucrose containing heparin. Micro hematocrits were done and 1 ml. aliquots were taken. The cells were centrifuged and washed once with isotonic sucrose. The cells were diluted to 200 ml. with water and this solution was used in order to determine the concentration of sodium and potassium of the cells.

FIGURE 4



Standard Curve for the Determination

of Cholinesterase Activity

RESULTS

Female albino rats were placed on the various diets and after they were depleted of vitamin E as shown by carrying out a dialuric acid hemolysis test (10), a minimum period of two weeks, they were sacrificed. The animals were killed by decapitation and the blood was collected into an isotonic saline solution containing heparin. The cells were centrifuged and washed twice with saline. Some of the cells were made up to the desired dilution for the dialuric acid hemolysis test and the rest were used in the cholinesterase determination.

The results from the experiments carried out on the normal and vitamin E-deficient animals are shown in Table II. The red blood cells of rats receiving a complete diet showed little or no hemolysis with dialuric acid while those from rats receiving a vitamin E-deficient diet showed extensive hemolysis when incubated with dialuric acid. The cholinesterase level of the erythrocytes was expressed in microliters of 1 N. acetic acid liberated by 0.1 ml. of cells from acetylcholine bromide. The cholinesterase level of the erythrocytes of vitamin E-deficient animals was found to be much lower than the cholinesterase level of the erythrocytes of normal rats. The t test of significance

showed the difference to be highly significant.

The effect of feeding other antioxidants, namely phenothiazineand two of its oxidized derivatives phenothiazone and phenothiazine-o-sulfoxide, are indicated in Table III. The result of feeding the three compounds falls into two categories: (a) a protective action similar to that of feeding alpha-tocopherol is shown, and (b) there is no protective action shown. Phenothiazine and phenothiazone fall into the first classification, while phenothiazine-o- sulfoxide falls into the second classification. The t test of significance indicates that there is a highly significant difference in the cholinesterase level of the erythrocytes of rats receiving a tocopherol-deficient diet and of those rats receiving a diet containing either phenothiazine or phenothiazone. The same test of significance revealed no difference between the cholinesterase activity of the red cells of rats on a tocopherol-deficient diet and the cholinesterase activity of the red cells of those rats receiving a diet containing phenothiazine-o-sulfoxide.

The determination of the sodium and the potassium content of the cells of normal and tocopherol-deficient rats is shown in Table IV. The results indicate that there is virtually no difference in the sodium and the potassium content of the erythrocytes of the various animals and

hence there is probably no drastic change in the cation concentration of the cell.

TABLE II

	% Hemolysis (Dialuric Acid)	ChE Detm'n* µl HOAc liberated from 0.1 ml. R.B.C.
NORMAL RATS	0.4 9.8 3.3 0 9.0 1.1 8.8 Average = 4.6 ± 4.4%	29 12 23 39 23 21 21 13 Average = 22.5 ± 3.04µl
VITAMIN E-DEFICIENT RATS	79 90 100 86 98 100 100 Average = 92 ± 8.6%	9 11 0 8 7 8 17 2 Average= 7.8 ± 1.6µl

Dialuric Acid Hemolysis and Cholinesterase Studies on Vitamin E-Deficient and Normal Rats.

* $t = 4.0$ for the two above groups

$p \leq 0.01$

TABLE III

Experimental State of Animal	% Hemolysis (Dialuric Acid)	ChE Detm'n * µl HOAc liberated from 0.1 ml. R.B.C.
Vitamin E-Deficient (8 rats)	93 ± 9.34%	7.8 ± 1.6 µl.
Phenothiazine Fed (6 rats)	1.7 ± 0.81%	25 ± 2.74 µl.[1]
Phenothiazone Fed (6 rats)	8.6 ± 4.1%	20 ± 3.56 µl.[2]
Phenothiazine-o-sulfoxide Fed (6 rats)	85.5 ± 11%	8 ± 2.62 µl.[3]

The Effect of Feeding Phenothiazine, Phenothiazone, and Phenothiazine-o-sulfoxide to Tocopherol-Deficient Rats.

* t values for these groups are

1. $t = 5.7$ $\quad P < 0.01$
2. $t = 3.1$ $\quad P < 0.02$
3. $t = 0.06$ $\quad P > 0.5$

TABLE IV

Rats	Na^+ ml.eq/liter of packed cells	K^+ ml.eq/liter of packed cells	Total Base	% Hemolysis (Dialuric Acid)	ChE Detm'n µl HOAc liberated from 0.1 ml. R.B.C.
Vitamin E-Deficient (3)	3.64	78.7	82.3	97.3%	9 µl
Normal (3)	3.37	84.9	87.9	6.3%	18 µl

Determination of Sodium and Potassium of Vitamin E-Deficient and Normal Rats.

DISCUSSION

Hemolysis *in vitro* of the erythrocytes of vitamin E-deficient rats can be accomplished by means of dialuric acid while the red cells of rats receiving vitamin E in the diet are protected from this hemolytic action. The results from the experiments performed are in complete agreement with the results obtained by György and Rose (8, 9, 10, 24).

The effect noted by us of feeding antioxidants other than alpha-tocopherol tends to bear out the results reported by Moore, Sharman, and Ward (19) who showed that some antioxidants were capable of replacing alpha-tocopherol in the diet. They found that thiodiphenylamine could prevent brown discoloration of the uterus while Dam and his coworkers (12) found that this same compound caused an increased deposition of vitamin A in the liver of rats on a vitamin E-deficient diet. Work in this laboratory has shown that thiodiphenylamine or, as it is often called, phenothiazine, will prevent the dialuric acid hemolysis of red cells of rats when it is included in the diet at a level of 0.126%. Both Dam *et al.* and Moore *et al.* added phenothiazine to the diet at a level of 0.068% which is a considerably smaller amount than that used

by us. A theory propounded by Moore, Sharman, and Ward (19) states that some degree of specificity, which is not a general property shared by a wide range of redox dyestuffs, is required by antioxidants for tocopherol-like activity. This theory could quite easily explain why phenothiazine and one of its oxidized derivatives, phenothiazone, could replace alpha-tocopherol in the diet, while another oxidized derivative, phenothiazine-o-sulfoxide, appeared to have little or no tocopherol activity. Just what the specifications for the anti-oxidant are in order to replace vitamin E in the diet is still an unknown factor.

The red cells of vitamin E-deficient animals, in addition to showing hemolysis with dialuric acid, present a rather interesting picture in regard to their cholin-esterase activity. Unfortunately the red blood cells of rats show a very low cholinesterase level and for this reason the results are perhaps not as conclusive as they might otherwise be. Therefore the cholinest-erase level of the red blood cells of mice, which were being killed for another experiment, was det-ermined in order to see if the cholinesterase activity of their erythrocytes might be higher. However mice fall into the same group as rats in that they also show

a low acetyl cholinesterase level in their erythrocytes. In general it would seem that the erythrocytes of tocopherol-deficient rats have a much lower cholinesterase level than do the red cells of normal rats. By carrying out the t test of significance it was found that there is a difference between cholinesterase activity of the red cells of those rats receiving a tocopherol-deficient diet and of those rats receiving alpha-tocopherol as a supplement to the diet. This result recalls the work of Bloch (21) who showed a correlation between alpha-tocopherol and acetyl cholinesterase in the brain and nervous tissue of the rat.

Vitamin E deficient rats which received the antioxidants, phenothiazine and phenothiazone, showed a cholinesterase activity in their erythrocytes corresponding closely to the values obtained from the red cells of normal rats. The rats receiving phenothiazine-o-sulfoxide on the other hand showed a cholinesterase level corresponding closely to the value obtained from the red blood cells of vitamin E-deficient animals. This finding would seem to show that if an antioxidant is capable of preventing some vitamin E deficiency symtoms such as dialuric acid hemolysis of the red blood cell *in vitro*, it is also capable of maintaing the cholinesterase level of the cell. This is particularily

true of thiodiphenylamine which has been shown to prevent several symptoms of vitamin E-deficiency (12, 13, 19). One wonders if there is a relationship between anti-oxidants and acetyl cholinesterase.

The studies on sodium and potassium content of the cells would seem to indicate that the cation concentration of the cell is not changed. In any case the difference must be so small that only by carrying out a large number of determinations could any statistically significant difference de demonstrated.

If, as the work done by us would indicate, the cholinesterase level of normal rats is extremely low, an interesting problem arises as to how the sodium and potassium concentration of the cell is maintained. According to Greig et al. (1, 26, 27, 28, 29, 30, 31, and 32) the cholinesterase activity is important in the maintenance of the cation gradient. If this hypothesis is accepted it does not explain that, although there is a drop in the cholinesterase level of the erythrocytes of vitamin E-deficient rats, the sodium and potassium concentration of the red blood cells appears to remain constant. It is true that the cation gradient could have changed because unfortunately, due to the tendency of rat blood to clot extremely easily, the sodium and potassium con-

centration of the plasma could not be obtained.

It has been shown, that although the sodium and potassium concentration of the cell remains constant, one enzyme system of the cell is changed when tocopherol is excluded from the diet and it is quite possible that other enzyme systems may also be affected. But it is still not known what the change in the red blood cell is which allows the erythrocytes of vitamin E-deficient animals to undergo hemolysis in the presence of dialuric acid.

SUMMARY

1. The results obtained show that the erythrocytes of the tocopherol-deficient animals may be protected from hemolysis *in vitro* with dialuric acid by the addition of some antioxidants to a tocopherol-deficient diet. The antioxidants which showed this protective action were phenothiazine and one of its oxidized derivatives, phenothiazone. Another derivative of phenothiazine, phenothiazine-*o*-sulfoxide, showed no protective action when it was included in the diet.

2. The cholinesterase level of the red cells of vitamin E-deficient animals was shown to be lower than the cholinesterase level of the red cells of rats receiving alpha-tocopherol in the diet. The inclusion of the antioxidants phenothiazine and phenothiazone in the diet as a replacement for alpha-tocopherol maintained the cholinesterase level of the red cell. Phenothiazine-*o*-sulfoxide failed to maintain this level of cholinesterase activity in the erythrocyte.

3. Despite a change in the cholinesterase activity in the erythrocyte the sodium and pot-

assium ion concentration of the red blood cells of vitamin E-deficient rats appeared to remain the same as that found in normal rats.

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